THE PREPARATION OF VARIOUS COMPONENTS OF COLLAGEN

by

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A b s t r a c t: Procedures were developed for the preparation and purification of the collagen fractions from experimental granulation tissue. One fraction which contained apparently intact α-, β- and x-components originated from insoluble collagen.

The x-components, which migrate behind the β-components, were isolated from the starch-gel sheets after an electrophoretic fractionation of the mixture. These components are easily degraded to the α- and β-components during the manipulations. Similar x-components can be purified from certain fractions eluted from a CM-cellulose column.

Several good procedures exist for the isolation of collagen from tissues which contain it as the main protein (1, 3—6, 9, 10). The preparation of collagen for analytical purposes from tissues, e.g. granulation tissue, which contain other proteins abundantly, remains a special problem, although various methods have been formulated for the isolation of collagen from experimental granuloma (2, 8), which has been frequently used in the studies on the formation of collagen. The preparation of the larger aggregates of tropocollagen, i.e. the γ-, δ- or x-units (15), in a pure form has been impossible thus far.

In this paper we present procedures for the preparation of the collagen fractions from sponge-induced granulation tissue, and for the isolation of the x-components from rat-tail-tendon collagen.

MATERIAL AND METHODS

Granulation tissue. — The growth of the experimental granulation tissue was induced by the implantation of four pieces (10 × 10 × 20 mm each) of viscose cellulose-sponge subcutaneously in the backs of albino rats (about 120 g). The granulomas were harvested 14 days after the implantation under ether anaesthesia, freed from the capsule and either stored frozen at —18°C or treated immediately with a Bühler-homogenizer (No. 210000, E. Bühler, Tübingen, West Germany). The further preparation of the collagen fractions is shown in Fig. 2.

Rat-tail-tendon collagen. — The tail tendons of rats (about 2 years old) were dissected, rinsed in cold water and blotted dry. Collagen, which is known to contain plenty of x-components, was obtained by shaking the fibres with acetic acid (0.5 *M*) overnight at +4°C. The solution was cleared by centrifugation for 60 min. at 35,000 *g* in a refrigerated centrifuge. The supernatant was lyophilized and the residue dissolved into 0.5 *M* acetic acid to contain about 0.6 % (w/v) of collagen.

Chemical analyses. — Nitrogen was determined as ammonia with conventional methods after Kjeldahl-combustion and hydroxyproline according to Stegemann (14) as modified by Woessner (16). The starch-gel electrophoresis was performed as described earlier (11).

Sedimentation analysis. — The solution of x-components (1.3 mg/ml) was dialyzed twice for 24 hrs and finally once for 12 hrs at +4°C against sodium acetate buffer, pH 4.8, ionic strength 0.15. Before the ultracentrifugation the sample was denatured for 30 min. at +37°C. The runs

with two collagen concentrations were performed with a Spinco E analytical ultracentrifuge at +37°C and 59,780 rpm. in a 12 mm aluminium cell. The $\bar{v}$ was assumed to be 0.700.

RESULTS

Extraction of collagen from the granulation tissue. — When homogenized granulation tissue was extracted either with NaCl-solution or with sodium acetate buffer at +4°C the supernatants contained plenty of non-collagenous proteins but little of collagen (Fig. 1). During extraction at

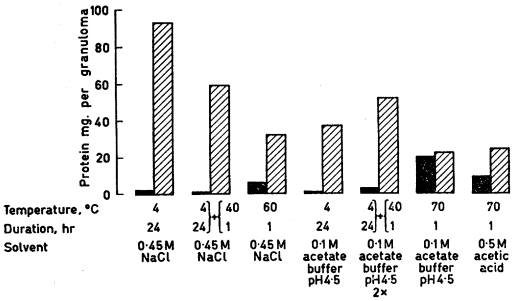

Fig. 1. — Dissolved collagen (black columns) and total protein (shaded columns) after the extraction of homogenized experimental granuloma pieces (à 2 cm³) with various solutions at the indicated conditions.

a higher temperature collagen is denatured and therefore more soluble, while the non-collagenous proteins are partly denatured and precipitated. This finding is applied in the procedure shown in Fig. 2.

Several solvents, including 20% (w/v) urea solution, were tried for the first removal of non-collagenous protein. Sodium hydroxide solution (1, 4) was found to be the best. Only a very small amount of hydroxyproline-containing material dissolved, but most of the non-collagenous protein did (Fig. 2). The major part of this hydroxyproline is probably not due to collagen but either to free hydroxyproline or small peptides. The urea solution extracted about 5% of the collagen and caused difficulties during the subsequent manipulations.

Collagen itself was extracted with acetic acid, first the soluble fraction at +4°C, and then a part of the insoluble collagen at +40°C. It is known that intact components of collagen can be released by gentle heating into soluble form from the conventional insoluble fraction (13).

Purification of collagen from granulation tissue. — Collagen was purified from the extracts with dialysis and precipitations with salts as described in the flow sheet (Fig. 2). The two final preparations contained 13.4% hydroxyproline and 18% nitrogen, and were regarded as pure collagen, which was confirmed by the gel-electrophoretic patterns (Fig. 3). The soluble collagen (Fig. 3C) yields more a-chains and fewer β-components than the fraction derived from insoluble collagen (Fig. 3B). A part of the insoluble collagen is left in the residue and it is dissolved only in degraded state at higher temperatures when the primary structures are also broken.

Preparation of the x-components. — One ml of the above-mentioned 0.6% solution of rat-tail-tendon collagen, dialyzed overnight against the electrophoretic buffer (sodium acetate buffer, pH 4.5, ionic strength 0.017) and denatured for 15 min. at +40°C, was imbibed in a strip (6.0×1.0 cm) of Whatman No. 4 paper. This piece of paper was placed in a slot which had been made in the starch-gel sheet (14.7% of hydrolyzed starch, w/v, in the mentioned buffer). The electrophoresis was carried out at 120 V for 3 hrs at room temperature to avoid the degradation of the collagen.

After the run the surface layer of the sheet (about 1 mm thick) was stained with

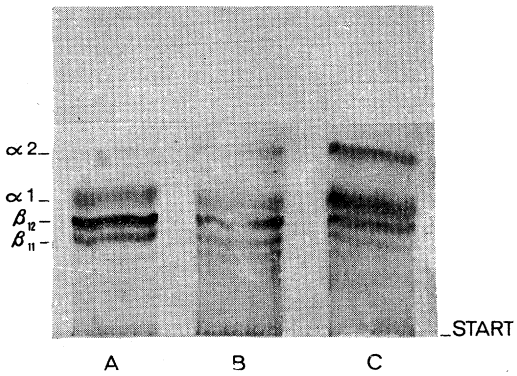

Fig 3. — Starch-gel electrophoretic patterns of denatured soluble collagen (C) and of an isolated fraction from insoluble collagen (B) from experimental granuloma, Sample A was extracted from rat skin with 0.45 *M* sodium chloride and purified.

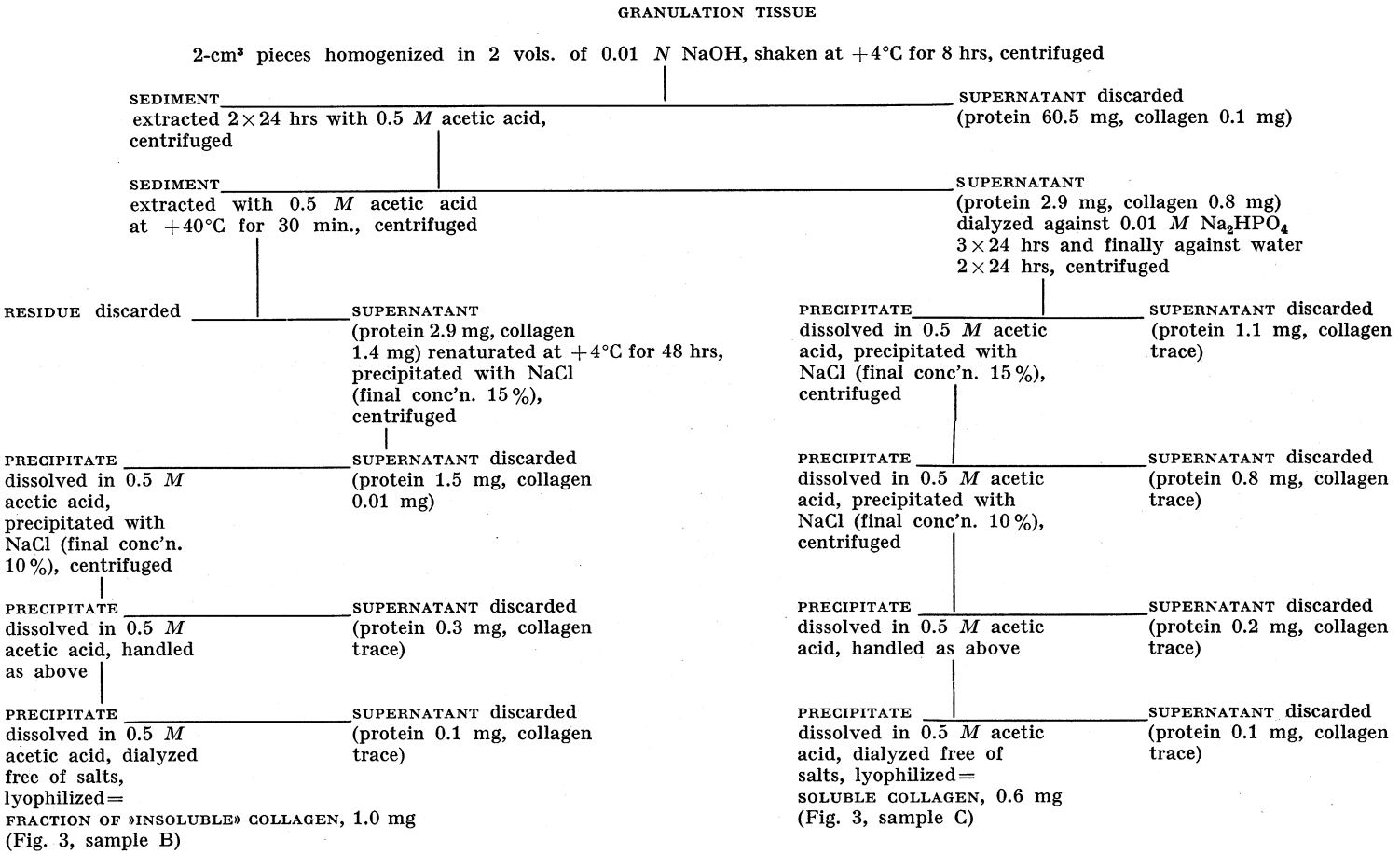

Fig. 2. — Flow sheet of the extraction and purification of collagen fractions from experimental granulation tissue. If not stated otherwise, all the manipulations were carried out at +4°C and the centrifugations performed with a refrigerated equipment for 30 min. at 35,000 *g*. The amounts are calculated for 2 cm³ pieces of granulation tissue.

0.1% solution of nigrosine in water-glycerol mixture (1:1). After the location of the *x*-components, the bands were cut from the sheet in one block, which was cast with 12% starch gel into the cathode-end of the electrophoretic bed (11) at a temperature lower than +36°C. At a higher temperature the *x*-components were rapidly lost. The cathode-end of the electrophoretic bed was now covered with a dialysis bag containing 20 ml of electrophoresis buffer. The *x*-components were driven from the gel to the buffer with an electric current (120 V for 3½ hrs). The contents of the dialysis bag were rinsed in a lyophilization flask with 0.5% acetic acid. About two thirds of the *x*-components could be harvested in this manner. Several lyophilized residues were combined, dissolved in 0.5 *M* acetic acid and the solution cleared at 35,000 *g* for 30 min. in a refrigerated centrifuge. The pellet contained starch but only a little collagen. The salts were removed from the supernatant employing a small Sephadex G-25 column. Several experiments were made with columns of CM-cellulose, CM-Sephadex and SE-Sephadex to remove the last traces of starch. A significant improvement was not obtained, because collagen was very tightly absorbed in these materials.

The gel-electrophoretic patterns of various preparations of the *x*-components are illustrated in (Fig. 4 A, B). They do not contain any α- or β-components, but the degradation already occurs easily after an extensive dialysis (Fig. 4C). This finding is in agreement with the experience of Hollmén and Kulonen (7) that the *x*-components are lost in preparative column electrophoresis, which takes a relatively long time. When the samples were analyzed with ultracentrifugation, they contained two shallow peaks with the sedimentation coefficients $s^{\circ}_{20,w}$ 3.36±0.30S and 4.34±0.10S, which agree with the values of α-and β-components given in the literature (12). When the material which had been used in the ultracentrifugation was studied again with starch-gel electrophoresis this conclusion was confirmed (Fig. 4D). The isolated *x*-components are in fact γ-components, but all three chains are not linked with covalent bonds.

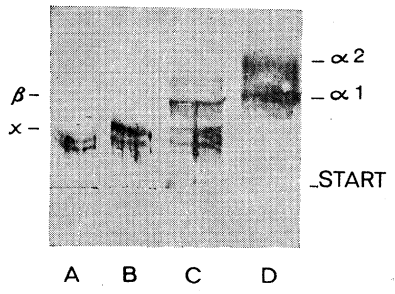

Fig. 4. — Starch-gel electrophoretic patterns of various preparations of the *x*-fractions. A and B = two different successfully prepared *x*-fractions. C=a similar sample as in B, but dialyzed exhaustively against pH 4.8 buffer for the sedimentation analysis, D=the same sample as in C, but after the ultracentrifuge run.

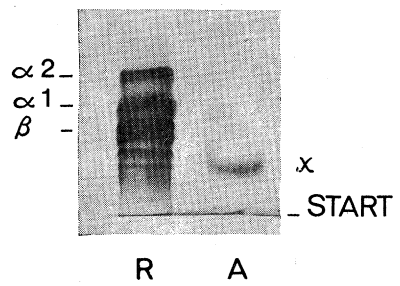

Fig. 5. — Starch-gel electrophoretic pattern of a *x*-fraction from CM-cellulose fractionation, purified with Sephadex G-200 column (A). R denatured soluble collagen.

The *x*-components can also be obtained in electrophoretically pure form with an another procedure (Fig. 5). When denatured rat-tail-tendon collagen is fractionated with CM-cellulose column as described by Piez et al. (12) the »tail» after the $\alpha_1+\beta_{11}$ complex contains the component γ_{111} (15). When this »tail», or rather »valley», was pooled and fractionated in Sephadex G-200 column (with acetic acid as eluant), two peaks were observed. The material in the first peak was pooled, lyophilized and analyzed by starch-gel electrophoresis. A single band, corresponding to the *x*-components, was observed.

DISCUSSION

The preparation of a soluble fraction from insoluble collagen with intact α- and β-components has a certain value in studies on the maturation of collagen, which is influenced, for example, by age and by intoxications like lathyrism.

The successful preparation of the x-components resulted in the conclusion that the x-components contain tropocollagen with the α- and β-components so loosely associated that they are detached by denaturation in mild conditions.

Collagen of rat-tail-tendon is exceptional, for example, in solubility. Further, in the γ-components from other tissues all three chains may be covalently bonded.

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